MICROBIOLOGY OF ORIENTAL FERMENTED FOODS

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INTRODUCTION

Many papers have been published on various aspects of Oriental fermentations used to produce food, but few have been devoted to the microorganisms used in the processes. This review will emphasize the kinds of microorganisms used and their utilization in mixed-culture combinations.

Mixed-culture fermentations in the preparation of foods are fairly common in the Western world, but noticeably moreso in the Far East. This review will be restricted to traditional fermented foods made in the Orient, including Japan, Indonesia, India, Pakistan, Thailand, Philippines, Taiwan, China, Korea, and the encompassing areas. These foods were produced long before written history; some of these processes are so little known that even today one can only guess as to the organisms used.

The fermented food industry in the Orient is large. Aiba (1) states that the value of fermented foods in Japan was $550,000,000 in 1974 as compared to the antibiotic industry of $1,183,000,000 or the production of ethanol at $27,000,000. The quantities of food produced by fermentation are great. The Japanese Food Agency, Ministry of Agriculture, Forestry, and Fisheries (15), gave the following figures for 1979: miso, 567,776 tons; shoyu, 1,252,431 kl; and natto, 158,000 tons. In Korea, 35% of the 442,803 metric tons of soybeans produced is fermented. Indonesia uses about 75,600 tons of soybeans in making tempeh. Recent data from the Soycrafters Association of North America state that a total of 926,640 pounds of tempeh (retail value $1,667,952) was produced in the United States in 1980 using *Rhizopus oligosporus* NRRL 2710, originally isolated from Indonesian tempeh. In Thailand, Bhumiratana (5) reports that a total of about 120,000,000 liters of nam-pla (fermented fish) was produced in 1977. The fermentation industry in the Orient is huge and is beginning to be transferred to the West.

HISTORICAL ACCOUNT

There is considerable ancient writing in Chinese publications about foods made by fermentation, but the first scientific reports are only about 100 years old. From 1878 until the beginning of World War I, there was an explosion of papers and reports dealing with fermented foods and drinks. For a discussion and references to some of these papers, see Hesseltine & Wang (27). In general, studies between 1881 and 1914 were devoted to the description of the product and the local name and to the isolation and description of the microorganisms associated with the fermentation. A number of organisms new to science were described and illustrated. Additional information was given on the action of the fungus on the substrate, suggested uses of the fungus in processes that could be exploited in European technology, and a description of the substrate preparation, food use, and native methods of food preparation.

This period of research ended abruptly with the advent of World War I, as the exchange of students and cooperation between Japan and Germany ceased. Food fermentation studies resumed in the 1950s and today considerable interest exists. This renewed interest stems from the concern with nutrition, the great enthusiasm for vegetarian and natural foods, the search for less expensive, high-protein foods, the influence of foreign students studying in the West, the need to expand export markets, the need to add products to convenience foods to add zest and flavor, and the interest in the activities of microorganisms used in fermented foods.

IMPORTANCE OF MIXED CULTURES

Relatively few genera and species of microorganisms are employed in the Oriental fermentations. Among the fungi, those used are restricted to *Rhizopus*,

Mucor, Amylomyces, Aspergillus, Monascus, and *Neurospora.* In this group, *Monascus* and *Aspergillus* are closely related, but the order *Mucorales* and the genus *Neurospora* are far apart in the classification of fungi. Note that yeasts often found are a special group of fungi closely related to *Aspergillus* and *Monascus,* all of which produce poorly developed ascocarps. All the fungi used are those that grow first in the succession of molds growing on plant material in nature. They therefore are the ones that rapidly use simple sugars. Before preservatives were used in bread, *Rhizopus stolonifera* was known as the "black bread mold" and *Neurospora* as the "pink bread mold." They were the first to grow in the microbial succession. Curiously, the fungi in the order *Mucorales* appear to be the ones domesticated in the tropics, whereas *Aspergillus* seems to be used more in temperate or semitropical countries such as China and Japan. The relative humidity of the areas may be an important selective factor. The yeasts are represented mainly by the genera *Saccharomyces, Candida (Torulopsis),* and *Saccharomycopsis* (22).

Among the bacteria, the lactic acid bacteria *Pediococcus, Leuconostoc, Lactobacillus,* and *Bacillus* seem to be the ones usually encountered.

In the Near East and Africa, molds seem to be little or never utilized; instead lactic acid bacteria and yeasts are the rule in the preparation of fermented foods (21).

Table 1 lists a number of Oriental fermented foods, the microorganisms used in their manufacture, and appropriate strain numbers of useful cultures.

In a number of fermented foods made in Asia, it is not clear what microorganisms are involved. For example, a Japanese fermented fish product, katsubushi, is processed by repeated fermentation steps using molds belonging to the genus *Aspergillus.* Little or nothing is known of the microorganisms involved producing the fermented fish sauces and pastes important in the Philippines and Indochina, except that they are halophilic bacteria. Some believe this digestion is more of an enzymatic process caused by the enzymes of the fish gut rather than the action of the microorganisms.

The microorganisms involved in a fermented food can be divided into the following categories.

1. Monoculture. Fermentations in which only one species of microorganism is necessary to produce the product. The Indonesian tempeh fermentation is an example: only *Rhizopus* is necessary to make the soybean food.

Natto is a food made by fermentation of soybeans with *Bacillus natto;* the whole fermented soybean, covered with a sticky polymer, is currently sold in plastic containers. The beans also may be coated with a salty covering and somewhat dried so that they are no longer sticky and can be kept for longer periods. Actually, only special strains of *Bacillus subtilis* are employed, and wise producers use a proven culture.

Ang-kak is a fermented rice product used for coloring other foods. The pigments are known and are water soluble. The only microorganisms used are

Table 1 Representative strains of cultures used in Oriental food fermentations

Food	Microorganisms used	NRRL number
Soy sauce	Aspergillus sojae	6271
	Aspergillus oryzae	1988, 6270, 24551
		1989, 24556
	Saccharomyces rouxii	Y–6681
	Candida etchellsii	Y–7583
	Candida versatilis	Y–7584
	Pediococcus halophilus	B–4243, B–4244
	Lactobacillus delbrueckii	B–445
Miso	Aspergillus oryzae	5593
	Aspergillus sojae	3485, 3486
	Saccharomyces rouxii	Y–11785
	Candida etchellsii	Y–7583
	Pediococcus halophilus	B–4506, B–4243, B–4244
Tempeh	Rhizopus oligosporus	2710
Sufu	Actinomucor elegans	3104, 2242
	Mucor dispersus	3103
Natto	Bacillus subtilis (B. natto)	B–4008, B–3383, B–3010
Tea fungus	Acetobacter sp.	B–2357
	Saccharomyces bisporus	Y–4810
	Candida sp.	Y–4882
Lao chao	Amylomyces rouxii	3160
	Rhizopus chinensis	3671
	Rhizopus oryzae	3142
	Saccharomycopsis fibuligera	Y–6720
	Saccharomycopsis malanga	Y–7067
Tape ketan	Amylomyces rouxii	5866
	Rhizopus chinensis	2870
	Saccharomycopsis fibuligera	Y–7145, Y–7170
	Saccharomycopsis malanga	Y–7175
Ang-kak	Monascus purpureus	2897
Ontjom	Neurospora intermedia	6025
	Neurospora intermedia	5506

selected cultures of *Monascus purpurea*. This is a primitive ascomycete producing simple ascocarps and eight-spored asci. Isolates sometimes are encountered in molding silage. Unpublished feeding experiments showed that isolates from silage are nontoxigenic. Producers indicate that most strains of this species are not suitable because they produce too little pigment.

Other processes requiring only one microorganism include the ontjom *(Neurospora intermedia)* and sufu *(Actinomucor elegans)* fermentations.

2. Multiculture. In these fermentations, more than one microorganism is required, belonging taxonomically to different species. An example of this is ragi, in which two fungi *(Amylomyces* and *Rhizopus)*, a yeast and a bacterium

are required to make the starter. (Ragi is not itself a food but is used as a starter for several food fermentations.)

3. Unimulticulture. These are fermentations in which two or more strains of the same species are used together. Soybean yogurt is an example in which two strains of *Lactobacillus acidophilus* are employed, with each strain contributing to the final product: flavor from one strain and acid from the other (33). In this fermentation, as in the multiculture fermentations above, two or more strains may be used.

4. Polyculture. These are food fermentations in which different microorganisms are many and the species specifically required to make the product are unknown. An example is the mixture of microorganisms found in silage fermentation and in Indochinese fish fermentations.

Except for the examples described above, in which only a single microorganism is employed even though there are usually several contaminating microorganisms present, most Asian food fermentations are carried out by more than one microorganism. The essential microbial cultures may be introduced simultaneously or they may be inoculated in sequence. To make lao chao, the starter culture containing the mold and the yeasts is added at one time. Sequential inoculation of microorganisms is exemplified in the shoyu fermentation, with koji first prepared using *Aspergillus oryzae* cultures, followed by a yeast-bacterial inoculation and then fermentation by the latter organisms. At this time the *Aspergillus oryzae* strains from the koji are killed.

Starter Cultures

One of the unique characteristics of the Oriental fermentations is the preparation of mixed culture inoculum produced as the starter for a number of fermentations. The starter in a dry state is prepared under relatively poor microbiological conditions by persons untrained or poorly trained in microbiology. These starter cultures are found in many countries in Southeast Asia, China, and the Indian subcontinent under such names as Chinese yeast, murcha, ragi, as well as a great number of Chinese names. The method of preparation and use of these starters go back centuries. By selection of mutant strains, certain organisms have been produced that do not exist elsewhere. Ellis et al (14) found *Amylomyces* and its single species *A. rouxii* to occur only in such starters. For a long time these organisms were believed to be *Mucor* fungi grown upon inadequate media; the genus *Amylomyes*, therefore was not thought really to exist. However, when ragi and Chinese yeasts were examined, *A. rouxii* was found to be present in most starters. Because it appears unable to survive in nature, this mold may have been selected as a mutant from the genus *Rhizopus*. The relationship to *Rhizopus* is based upon the fact that the colonies grow very rapidly, are white in color, and have abortive sporangia. *A. rouxii* sporangia take many forms. Some are mere spheres of protoplasm with

a sporangia wall at the end of a sporangiophore. Others have irregular differentiations of sporangiospores, but all cleavage is incomplete. Still others show irregular and angular spores varying greatly in size and shape, such as one would expect to encounter in an induced mutant of *Rhizopus*. Because of rhizoids, stolons, and black-pigmented sporangia, it is obviously derived from *Rhizopus* and probably closely related to *R. oryzae*. However, it fails to react sexually with *Rhizopus* or with different strains of itself.

Reproduction undoubtedly is due to the chlamydospores, because many strains fail to form any sporangiospores. Cultures are difficult to maintain because there are no sporangiospores to be used in lyophilization.

The ragi starter preparations take the form either of small hard powdery balls or of flattened round pieces of a floury material about 2–3 cm wide. The base material is rice flour. A second and more sophisticated form is a powdered preparation sold in sealed cellophane bags, much as baker's yeast is merchandised in the West.

Ragi and Chinese yeast are not foods in themselves but are starters consisting of rice powder with certain spices in which the proper organisms are present. In China and Southeast Asia, this powder is used to start food fermentations. Two foods made from this starter are the Indonesian tape (10) and the Chinese dessert lao-chao (45). Lao-chao, a typical fermentation using the Chinese yeast, will serve as an example. Lao-chao is prepared simply by soaking glutinous rice in water overnight and steaming it until the rice is soft but cooled. Some of the Chinese yeast powder is then sprinkled on the rice and thoroughly mixed in. The container of rice is incubated in a cool place. After a short fermentation (48 h), characterized by liquefaction of most of the rice to a clear semiliquid, the product is cooled. The food is then ready to eat as a dessert. It has a sweet, slightly fruity taste (somewhat like Juicy Fruit® chewing gum) flavored with alcohol. The *Amylomyces rouxii* and the *Rhizopus* strains break the starch down to sugar, which then can be fermented by the two species of yeast present, *Saccharomycopsis fibuligera* and *S. malanga*. (Note that the yeasts in lao-chao fermentation are not *Saccharomyces cerevisiae* but belong in the genus *Saccharomycopsis*. *S. malanga* and *S. fibuligera* were identified by yeast curator Dr. C. P. Kurtzman, who compared the ragi isolates against the type strains of those species.) In samples of murcha we have recently studied, *Amylomyces* and *Rhizopus* have been replaced with species of *Mucor*, probably because in the ancient past these species adapted better to the low temperature found at the sites of production. The two yeasts add flavor, small amounts of alcohol, and probably other substances to the product.

In addition to the Mucoraceae and the yeast, a third group of organisms are always present and seem to occur almost as pure cultures. These appear to be lactic acid bacteria, which occurs in counts of as high as 7 or 8×10^6 per gram.

A recent study of ragi (18) reported counts of bacteria up to 5.0×10^4 and identified the 17 strains isolated from ragi as *Pediococcus pentosaceus* Mees.

Besides the microorganisms and the starch rice powder, certain spices are always incorporated, consisting of garlic (*Allium sativum* L.), ginger (*Zingiber officinale* Rosc.), langkuas (*Alpinia galanga* Sw.), *Citrus aurantium L.*, and pepper (44). These spices probably inhibit contaminating organisms and stimulate the useful organisms in ragi. Spices, especially garlic, cinnamon, and cloves, are known to have antimicrobial activity (16). Kissenger & Zaika (34) reported that black pepper, allspice, and nutmeg stimulated the production of acid by *Lactobacillus plantarum* and *Pediococcus cerevisiae* mixtures but did not stimulate bacterial growth. In a further paper, Zaika & Kissenger (49) cite 13 references describing the inhibitory effect of spices on microorganisms. Soedarsono (44) reported that garlic greatly inhibited growth of *Aspergillus niger* and *Bacillus subtilis*. On the other hand, *Rhizopus* and *Amylomyces* were only slightly inhibited by garlic and, therefore, would grow in the ragi cakes. Ginger, likewise, did not inhibit *Amylomyces* or *Mucor*. This author concludes that certain spices inhibit many undesirable microorganisms. Thus, the rice flour–spice mixture acts as a selective medium for the growth in the flour of amylolyte-, alcohol-, and acid-forming microorganisms. A closer control of the amounts of inoculum, spices, and flour should produce a better starter which, in turn, would give a more uniform fermentation product. The alcohol content, flavor, appearance, and length of fermentation would be constant.

The method of preparing ragi is described by Saono et al (41). Ragi is made from rice flour mixed with finely ground garlic, black pepper, chili pepper, and *Alpinia galanga* (langkuas). Each factory uses differing proportions of ingredients. Enough water is added to make a thick paste, which is kneaded into small flattened balls. Next, powdered old ragi is sprinkled over the balls, which are then placed on a muslin-covered bamboo tray and air dried for 2–5 days at 25–30°C. Presumably, microorganisms develop at this time. Of course, there are modifications. In one, the ragi balls are placed over steaming palm sugar syrup for a few minutes prior to inoculation.

Djien (unpublished) gives further details on the manufacturing of ragi as a small cottage industry. The inoculum is added to the mixture of spices and glutinous or common rice flour before water is added, he states. During incubation, the desired microorganisms grow. Then the ragi balls are dried in the sun or air dried at room temperature. The balls or cakes remain active for several months during storage at room temperature. Traditionally, ragi balls are sold by the piece in shops and drugstores. Recently they have been packaged in cellophane bags of ten cakes each.

As an example of how ragi and similar products are used to produce food, Djien (10) has given a detailed account of the use of ragi to produce tape, a

popular Indonesian delicacy. Consumed as a snack or as a dessert, tape has a sweet acid taste with a mild alcoholic flavor and is partially liquified. The starting material may be glutinous rice *(Oryza sativa* var. *glutinosa),* which makes tape ketan or cassava *(Manihot utilissima)* to make tape ketella. Both products are produced on a home-industry scale or in the home. According to Djien, to make rice tape 100 g of rice is soaked in 150 ml of water overnight, then steamed in a rice steamer for 15 min. The rice then is broken up, wetted with 25 ml of water, and steamed again for 15 min. After transferring the rice to a bamboo basket covered with banana leaves, it is cooled and inoculated with ragi powder. The inoculum is mixed thoroughly with the rice. The rice is incubated at 30°C for 30 h, at which time the pH remains more or less constant at 4. The maximum reducing substances coincided with the fermentation reaching pH 4. Cronk et al (9) add that the fermentation may run 24–48 h at temperatures of 25–30°C. They report that about 2.7% vol/vol of ethanol was formed in 48 h. In the case of cassava, the roots are peeled and washed and the surface is inoculated.

In food fermentations other than ragi the microorganisms are pure culture spore or cell preparations that are packaged and sold either as a single strain or as a blending in the proper proportion of several strains. These processes have been reviewed in detail (26).

Mixed Pure-Culture Fermentations

Interest in mixed-culture fermentations is growing. *Mixed-culture Fermentations* (8), published recently as a special book of the Society of General Microbiology, describes the use of mixed cultures in brewing, biomass production, yogurt, and food fermentations. The use of mixed cultures is the rule, not the exception, in Oriental food fermentations. This has come about by the selection over the centuries of certain microorganisms that can grow together to produce a desirable product free from toxins and infectious organisms. This was done unknowingly by establishing ecological conditions that allowed the useful organisms to complement each other's activities and to live in harmony. The mixed-culture fermentations combining yeast and lactic acid bacteria are the most common. Wood (46) has summarized these types and lists the following as examples: sake, lambic beer, Geuze beer, Stock beer, ginger beer, whiskey, sour mash bourbon, kvass, kaffir beer, koumiss, kefir, tape, sour dough breads, Parisian barm bread leavan, soy sauce, miso, and a number of other fermented soy products.

In mixed fermentations, the substrate may take a variety of forms besides the liquid. A classificaton of fermentable material in which solid material is incorporated was published recently (24). Solid-state fermentations are applied to material that is in a solid state but may be in different physical states in relation to the whole fermentation menstruum:

1. Solid material only: (*a*) solid material allowed to ferment in place, as in making tempeh and natto; (*b*) solid material occasionally stirred, as in the koji-making process for miso and shoyu; and (*c*) solid material continuously agitated, as in producing certain mold metabolites, such as aflatoxin.
2. Solid material in liquid: (*a*) solid material in columns with liquid circulated through it, as in certain processes for producing alcohol; and (*b*) solid material suspended in liquid medium, either with agitation or stationary, as in kaffir beer and shoyu fermentations.

Mixed or multiple cultures have a number of advantages over conventional pure fermentations:

1. Enhanced yield. Driessen (11) studied the behavior of the mixed yogurt cultures *(Streptococcus thermophilus* and *Lactobacillus bulgaricus)* grown together and grown separately in the same medium. The two cultures separately produced 24 and 20 mmol ml^{-1} of acid, whereas when the same two strains were used together at the same inoculation rate, the yield was 74. Likewise the number of *S. thermophilus* cells increased from 500×10^6 per ml to 880. These studies were made on 3-h cultures incubated at 45°C. The number of *L. bulgaricus* cells, however, did not increase.
2. Protection against contamination. Pure cultures are always more subject to contamination than mixed cultures. In nature, a pure culture state rarely, if ever, occurs. Typically, in an ecological situation, one organism attacks the primary organic source but secondary organisms utilize the by-products. *Aspergillus oryzae* attacks the starch with its amylolytic enzymes, and the glucose is used in the second stage by yeast and bacteria in the shoyu fermentation. In such a mixture, especially if the inoculum is heavy, contaminants find it almost impossible to develop, at least to any . significant degree. Often the normal flora is overcome, even when the substrate is not sterilized.
3. Higher growth rate. As noted above, in mixed culture, the various microorganisms may produce needed growth factors or produce essential growth materials either as carbon or nitrogen sources beneficial to the second organism. This promotes better growth of the second organism and results in better formation.
4. Stable association of organisms. Even when mixed cultures are prepared by those untrained in microbiology, mixtures may be remarkably stable even after years of transfer. Ragi, prepared under completely unsterile conditions, has been made for centuries with the same three types of organisms still present. Probably by trial and error, the ragi makers learned the right cultural conditions for keeping the organisms together and passed this down from ragi maker to ragi maker. In pure-culture fermentations,

where high cell densities are maintained, there is often a lack of stability of the producing culture.

5. Products made by the mixture complement each other. In many of these associations, various products are produced that complement each other to the exclusion of other microorganisms. Yeasts produce alcohol and lactic acid bacteria produce acids; the growth of these organisms leads to the development of an anaerobic condition that excludes most molds and bacteria.

6. Mixed substrates. Substances used for food production are never pure compounds. Therefore, mixed cultures possessing a wider range of enzymes are able to attack a greater variety of compounds. They are better able to remove or change inhibitory constituents from the substrate, such as undesirable compounds in soybeans (e.g. trypsin inhibitors).

7. Multistep transformations. Mixed cultures can bring about a whole set of transformations that would be impossible for a single organism. Thus, in the miso fermentation, the *Aspergillus oryzae* strains used in the koji made from rice convert the starch to sugar; the yeast, in turn, uses the sugar for making small amounts of alcohol and other flavoring substances.

8. Mixed cultures can be maintained by untrained people. If the environmental conditions are constant (such as the unvarying temperature found in Indonesia) and the substrate is constant, it is relatively easy to maintain mixed cultures and carry out successful fermentations.

9. Phage. In pure-culture fermentations, especially with bacteria and actinomycetes, a problem that always arises during commercial production is the contamination of the developed culture with phage, which can completely wipe out production. Mixed cultures have a wider range of genetic resistance to phage; in such cultures, as far as I am aware, no failures due to infection of phage have been reported.

10. Simultaneous changes occur. In mixed fermentations, several different transformations of the substrate may occur at the same time, with the total not being posssible for any one organism. Thus, in the koji starter several *Aspergillus* strains are often mixed, with one strain producing high levels of amylases and a second producing specific proteases found in low levels in the first strain. The result is a mixture of desirable enzymes that can act on soybeans. This set of enzymes would never be found in a single strain. The advantages of mixed culture fermentation, as it applies to the methane single-cell protein fermentation, can be found in articles by Harrison (19, 20). A fascinating paper on mixed-culture fermentation, "New dimensions in microbiology: an introduction" (7), traces the history of pure-culture fermentations and the effect of this technique on the use of mixed cultures.

Some disadvantages to using mixed cultures should be pointed out. The product may vary more than would be expected for a pure-culture fermentation owing to the varying amounts of individual organisms in the inoculum. It is more difficult to study mixed-culture fermentations because of the interaction of two or more cultures. Defining the nature of the product and the microorganisms involved for patent and regulatory purposes is more difficult.

The use of mixed cultures offers an alternative to trying to incorporate new genetic factors into a single organism. For example, in making large amounts of ethanol from a starchy substrate, attempts might be made to incorporate genetic material from *A. oryzae* into *Saccharomyces cerevisiae*, so that one *S. cerevisiae* could both produce high levels of amylase and ferment this to high levels of ethanol. The other possibility would be to use two microorganisms, either together or sequentially, to make the product. Such a study has recently been made by Jones & Greenfield (32) on the production of high yields of ethanol from a concentrated substrate. Two yeasts were used: one was *S. cerevisiae*, which can be grown in a high substrate concentration but has low alcohol production; the second, a sake yeast, has a high tolerance to ethanol but a low tolerance to high substrate concentrations. When fermentations were run at high glucose levels, improved yields were obtained. Jones & Greenfield state that "the situation in which performance is most improved is where an ethanol-tolerant organism, which is limited by poor performance at high substrate concentration ratios, is mixed with an osmophilic strain with low to average ethanol tolerance." Increase in productivity was stated to be 10–20%.

Sequential Fermentations

In the above discussion, mixed culture fermentations were described in which the various microorganisms were added at the same time. However, great quantities of both alcoholic beverages and food are produced in fermentations, in which the cultures are added one after the other. Three of these, the shoyu, miso, and sake fermentations, each of which uses pure cultures as inoculum, have been widely studied. In each, the inoculum is in a pure form, from carefully selected and tested strains. It is extremely important that the cultures employed be correct. The use of the wrong microbial strain can lead to disastrous results:

1. Undesirable flavors and appearances may be produced. In extreme situations, growth may be greatly reduced or absent.
2. The culture can be an infection-causing pathogen.
3. In some instances, the wrong organism may produce a toxin. The use of *A. flavus* for *A. oryzae* can result in the formation of the highly carcinogenic compound aflatoxin.

4. An incorrect strain may lead to undesirable changes in the product, owing to enzymatic action on the substrate.

5. Contamination of the starter may lead to spoilage of the product. If koji becomes too moist, the substrate will become a wet, unmolded, stinking mess, quite unfit for use in the secondary fermentation.
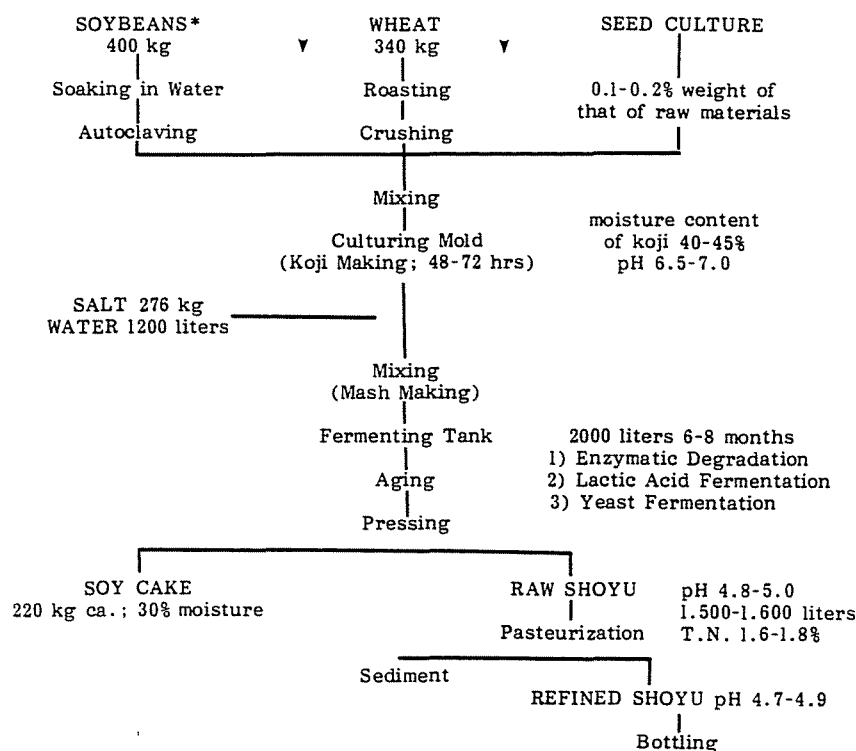
Anyone wanting to make Oriental fermented foods must use known, tested, pure strains and be able to recognize contamination. Most fermentation failures can be traced to a lack of appreciation for the importance of the inoculum and its handling.

SHOYU (SOY SAUCE)   The Japanese name for soy sauce, shoyu, implies that it is made by fermentation rather than by chemical treatment of soybeans. The following information about the microorganisms used in shoyu fermentation is taken mainly from a recent review by Yokotsuka (48), who cites a great deal of the recent extensive literature. Shoyu, a popular liquid condiment with an annual production of about 1.2 million kl is consumed at about 10 liters per capita per year in Japan. Shoyu is now sold worldwide. A growing amount of shoyu is being produced in the United States by fermentation. The Japanese Agricultural Standard (JAS) defines shoyu as made from heat-treated soybeans and wheat cultured with the koji molds, *A. oryzae* and *A. soyae*. The koji is mixed with salt water to make a mash, moromi. The moromi is fermented with yeast and lactobacilli and then aged. JAS recognizes five types, of which the Koikuchi type represents 85% of all the shoyu consumed in Japan. Good-quality Koikuchi shoyu has the following characteristics:

(*a*)1.5–1.8% total nitrogen (one half must be free amino acids): (*b*) 3–5% reducing sugar (mainly glucose); (*c*) 2–2.5% ethanol; (*d*) 1–1.5% polyalcohol (primarily glycerol); (*e*) 1–2% organic acid (primarily lactic); (*f*) 4.7–4.8 pH; and (*g*) 17–18% sodium chloride. Figure 1, taken from Yokotsuka (48), illustrates the sequential addition of fermentation microorganisms.

For making koji, wheat kernels are roasted at 170–180°C for a few minutes, then coarsely crushed. Whole soybeans or defatted soybeans grits are moistened and cooked with steam under pressure. Today, defatted flakes are generally used. These two materials are mixed and inoculated with seed mold *(A. oryzae* and *A. sojae)*. The molds are especially selected for high enzyme activity and the ability to grow rapidly in very thick substrates. The depth of the fermenting mass is 30–40 cm. Koji fermentation is typically carried out on a large perforated stainless-steel plate, 5 × 12 m for 2–3 days. Temperature and moisture levels are carefully controlled: 30°C, 40–43% moisture, with the moisture decreasing to 25–30%. This first fermentation is to produce desirable enzymes.

Yokotsuka states that good koji fermentation should: (*a*) give good flavor to the product; (*b*) produce a good amount of spores; (*c*) have a high percentage of

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SOYBEANS*              WHEAT              SEED CULTURE
    400 kg        ▼    340 kg    ▼             |
     |                   |                     |
Soaking in Water     Roasting          0.1-0.2% weight of
     |                   |              that of raw materials
 Autoclaving         Crushing                  |
     |_____  |  _____ |
                       Mixing
                         |              moisture content
                  Culturing Mold        of koji 40-45%
              (Koji Making; 48-72 hrs)     pH 6.5-7.0

  SALT 276 kg _____   |
WATER 1200 liters           |

                       Mixing
                   (Mash Making)
                         |
                  Fermenting Tank    2000 liters 6-8 months
                         |           1) Enzymatic Degradation
                      Aging          2) Lactic Acid Fermentation
                         |           3) Yeast Fermentation
                     Pressing
        _____|_____
       |                                   |
   SOY CAKE                           RAW SHOYU     pH 4.8-5.0
220 kg ca.; 30% moisture                  |         1.500-1.600 liters
                                    Pasteurization  T.N. 1.6-1.8%
        _____ |
       |                                  |
   Sediment                       REFINED SHOYU pH 4.7-4.9
                                          |
                                      Bottling
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* or Defatted Soybean 330 kg, moistened with 420 liters hot water

Figure 1   Koikuchi shoyu fermentation [from Yokotsuka (48)].

spores that germinate and grow rapidly; (d) have high enzymatic activity; (e) have good genetic stability; (f) give color to the product; (g) produce no mycotoxins; and (h) produce mash that is easy to press. Mutation has been used to improve strains of the koji mold. Inoculation of the wheat and soybean is at the rate of 0.1–0.2%. The incubation temperature for maximum mycelial growth is 30–35°C, followed by a lowering of the temperature to 20–25°C for maximum enzyme production—all of which is done in a 48-h period.

The second fermentation step is preparation of the mash, mixing salt water (22–23%) and 120–130% volume to the koji. The moromi is then transferred to deep fermentation tanks of 50–300 kl, now usually resin-coated iron tanks. The moromi is held for four to eight months, depending on the temperature, with occasional mixing with compressed air to ensure uniform blending and to promote microbial growth. Although the mold in the koji is killed, their enzymes hydrolyze most of the protein to amino acids and low-molecular-weight peptides. About 20% of the wheat starch is used by the koji molds, and

the remainder is converted to simple sugar, of which more than one half is fermented to lactic acid and alcohol by the lactobacilli and the yeast, respectively. Initially, lactic acid is produced, followed by the yeast fermentation. Pure cultures of selected strains of *S. rouxii* and *Pediococcus halophilus* are added to the mash at the beginning of the moromi mash fermentation. These organisms are protected from contamination to a large degree by the high salt in the moromi and lack of aeration. In addition to *S. rouxii, Candida etchellsii* and *C. versatilis* also may occur in the moromi and add flavor.

Following fermentation, the liquid is pressed from the moromi, refined, pasteurized, and packaged. Pasteurization is necessary to inactivate the enzymes and to kill the yeast bacteria population.

It is now a practice to add cold water to the koji to keep the new mash below 15°C for several days and then to gradually warm it to 28–30°C for 20–30 days. The lower initial temperature prevents the rapid decrease in pH and consequent inactivation of the alkaline protease of the koji molds. The major lactobacillus is *Pediococcus halophilus*. This organism is added at the rate of 10^{-2}–10^3 ml, and in three months reaches 10^6–10^7 ml. *Torulopsis* (now = *Candida)* is believed to be needed to give a good volatile flavor to the finished product.

MISO   Miso is like shoyu, but the finished product is a paste similar to peanut butter, varying from light yellow to almost black. There are a number of different kinds of miso, as of shoyu. Miso is typically used as a soup base but may be used to add flavor to foods such as fish, vegetables, shellfish, and meat. Two reviews on the subject have been published in English, by Ebine (12) and Shibasaki & Hesseltine (43).

Miso can be classified into three types, based on the substrate: rice miso, made from rice, soybeans, and salt; barley miso, made from barley, soybeans, and salt; and soybean miso, made only from soybeans and salt. These three are in turn classified on the basis of taste into sweet, medium salty, and salty. Unlike shoyu, whole soybeans are used because defatted flakes make an inferior product. Soybeans are soaked in water, then cooked in water at 115°C for 20 min. The koji is made from cleaned milled rice that is soaked in water and then steamed in an open cooker for 40 min. About 80% of the miso production in Japan is the rice koji type. The cooled rice is then sprayed with tane koji spores of *A. oryzae*. Tane koji refers to the dry spores often prepared by a factory engaged only in inoculum production. The rate of inoculation is 1 g of a tane koji preparation containing 10^9 or more viable spores per kg of raw rice. In some instances, only one strain of *A. oryzae* or *A. sojae* is used, but in many instances, as in the shoyu koji, as many as three strains of mold are blended together. The inoculum is conidia only, since no other means of reproduction is known in these species. Typically, the koji mold consumes about 10% of the rice. The koji is fermented for 40–48 h in a variety of different types of

koji fermentors. Salt and cooked soybeans are added to the rice koji; the salt, plus the anaerobic conditions developing in the second fermentation, kills the molds. As is not the case in preparation of shoyu, in that of miso no water is added; therefore, the fermenting mash is a thick paste. At the time of mashing, a pure culture of yeast and lactic bacteria is added. Fermentation proceeds slowly at 30°C and continues for one to three months, depending on the variety of miso desired. Since the mash is a paste, it cannot be stirred with air, but rather the immature miso is removed from one vat to another at least twice to improve fermentation. Typically, the yeasts are *Saccharomyces rouxii, Candida versatilis,* and *C. etchellsii;* the lactic bacteria are *Pediococcus halophilus* and *P. pentosaceus.* This does not exclude certain other halophilic yeasts and bacteria from growing in or on the fermenting paste. The inoculum is typically at the level of 10^5 per g. A typical fermentation will yield 3300 kg of miso with a moisture content of about 48% obtained from 1000 kg of soybeans, 600 kg of rice, and 430 kg of salt. The final product has the following characteristics brought about by the koji enzymes and the action of yeast and bacteria: 60% of the protein is water soluble, including amino acids and polypeptides; 75% of the carbohydrate is reducing sugar; and trypsin inhibitors and hemagglutinins are inactivated. The product is safe to store for long periods because of the high salt content (6–13%). However, in making miso soup, it is a practice to add ten times the weight of miso with hot water to give a salt content of about 1.2%. After aging, the miso is packaged and sold as a paste, but it also may be dehydrated. Much work is being done to reduce the salt content and to make miso with enzymes derived from molds.

SAKE   Sake is the traditional national drink of Japan as wine is that of France. The following account is taken from a review article in English by Imayasu (30), who states that sake has four unique characteristics: (*a*) In Japan, sake preparation is based on fermentation of nonglutinous rice, whereas elsewhere in Asia it is made from glutinous rice. (*b*) Koji is used for saccharification. (*c*) Saccharification and fermentation occur at the same time. (*d*) Sake has the highest amino acid content of all alcoholic drinks. Amino acids come from the enzymatic digestion of rice protein by the mold enzymes.

The process of sake brewing is outlined by Imayasu (30) in Figure 2. Soaked rice is steamed and cooled, and mold spores of appropriate *A. oryzae* strains are used to inoculate the rice. The incubation has humidity control and is held at 35°C for 35 h. The moldy rice is mixed with water, steamed rice, and a sake yeast culture. This material becomes the seed mash or "moto." The seed mash is a pure yeast culture in concentrated form used to start the main fermentation, and additional seed mash is added to the main fermentation.

The koji molds bring about saccharification of the rice starch, and the sugar thus formed is fermented into alcohol by the sake yeast. The yeast is called
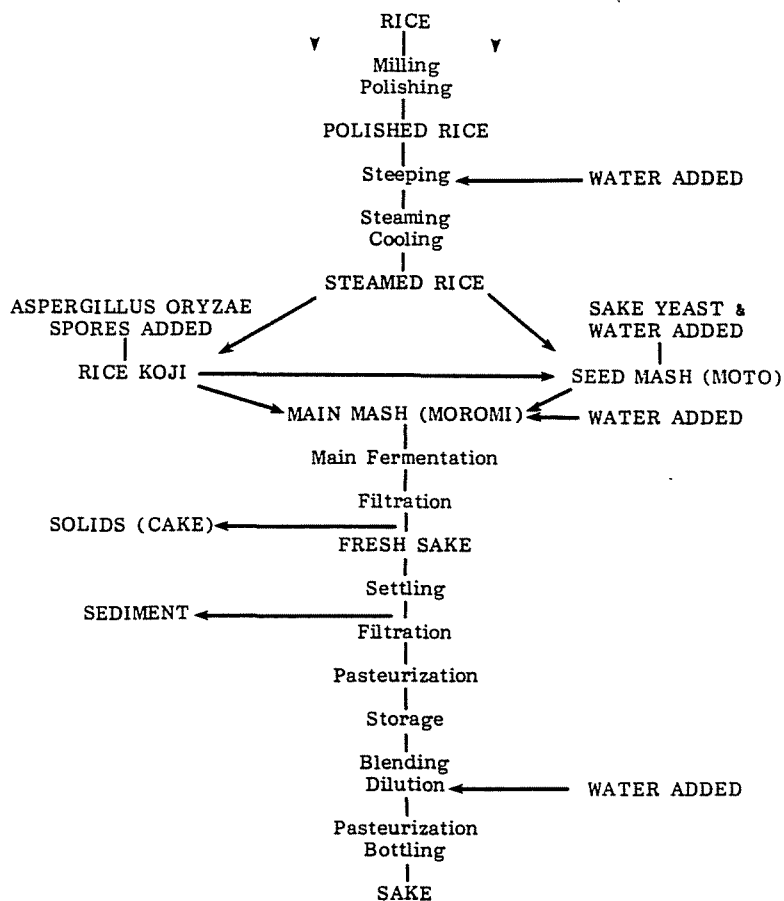
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                              RICE
                ▼             |            ▼
                           Milling
                           Polishing
                              |
                        POLISHED RICE
                              |
                        Steeping ◄──────────WATER ADDED
                              |
                        Steaming
                        Cooling
                              |
                        STEAMED RICE
  ASPERGILLUS ORYZAE     ╱              ╲      SAKE YEAST &
    SPORES ADDED        ╱                ╲     WATER ADDED
        |              ╱                  ╲        |
    RICE KOJI ──────────────────────────────► SEED MASH (MOTO)
              ╲
               ╲► MAIN MASH (MOROMI) ◄── WATER ADDED
                       |
               Main Fermentation
                       |
                   Filtration
  SOLIDS (CAKE) ◄──────────|
                   FRESH SAKE
                       |
                   Settling
   SEDIMENT ◄──────────|
                   Filtration
                       |
                 Pasteurization
                       |
                   Storage
                       |
                  Blending
                  Dilution ◄──────── WATER ADDED
                       |
                 Pasteurization
                   Bottling
                       |
                     SAKE
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Figure 2 Processing chart for Japanese sake [from Imayasu (30)].

Saccharomyces sake but, according to modern classification, is *S. cerevisiae*. The average yield of sake is 3 liters per kg of rice.

Recent improvements of the microbiological aspects of the sake fermentation has involved five changes: (*a*) Instead of adding lactic bacteria to the sake yeast culture to control bacterial contamination, now measured amounts of commerical lactic acid are added. (*b*) Because of the use of pure culture yeast inoculation, production of sake is carried out without sterilization in open tanks. (*c*) Nonfoaming yeasts, which have all the other desired characteristics, have shortened the fermentation time and reduced the number of tanks needed. (*d*) Commercial enzyme preparations of mold origin are partially used to convert the rice starch to sugar. (*e*) Continuous fermentation, with a series of four open tanks with an overflow system, or a closed continuous single fermentation is possible. In the latter system, large quantities of pure culture

yeast must be added (10^9/mol) rather than moto. As one can see in Figure 2, microorganisms are used sequentially, with koji formed separately to add to the mash, followed by alcohol fermentation carried out by the sake yeast.

MICROORGANISMS USED

A number of steps are required to determine the types of the microorganism(s) responsible for traditional fermentations: (a) Information must be obtained on the method used by the local people to prepare the food, such as moisture content of substrate, temperature of incubation, and time of fermentation. (b) The dominant microorganism(s) must be isolated from at least ten fresh good samples collected from different locations or factories. (c) Each of the isolates must be tested by producing the native product in the traditional way. (d) Cultures that produce the proper product should be saved. (e) Proven producing strains must be studied comparatively, a description covering the variations observed, a comparison made with type cultures or descriptions, and a scientific name assigned. (f) Several producing strains should be preserved permanently in a culture collection to allow further studies on their enzymes, toxicity, growth requirements, vitamin formation, and other biological activities, and for improvement or modification of production methods.

Zygomycetes

From studies thus far on fermented foods of the Orient, the lowest group of fungi in the evolutionary scale found is the Mucorales; four and possibly five genera involved belong in the family Mucoraceae. These may be separated on the basis of a generic key [(25), modified and shortened here] (see Key 1).

RHIZOPUS EHRENBERG   This genus is in need of further study. It is represented by many species that integrate with each other. The classification of *Rhizopus* species and an account of earlier classification of the genus may be found in Inui et al (31). These authors recognize 14 species, but the number of species described in the literature may run to 100. A more recent treatment of the genus by Zycha & Siepmann (50) recognizes only ten species in the entire genus. Species found in ragi, Chinese yeast, tempeh, and koji fall within about four groups of species. These groups are closely related and may represent variations of only one species. For help in separating the four species found in specific foods see Key 2.

MUCOR MICH. EX L.: FRIES   Species of the genus *Mucor* are found in such varied fermentations as sufu (Chinese cheese), ragi, murcha, and Chinese yeast. *Mucor* is recognized by its rapid growth, production of multispored globose sporangia, lack of rhizoids and stolons, and spreading colonies from

Key 1 Identifying genera of Mucorales involved in food fermentations

Identifying characteristics	Genus	Or see characteristic number
1a Sporangia poorly developed or abortive, aerial, and substrate producing innumerable chlamydospores	*Amylomyces (Chlamydomucor)*	
1b Abundant sporangia produced; chlamydospores present or absent		2
2a All sporangia pyriform and with an apophysis	*Absidia*	
2b Sporangia globose		3
3a Sporangia borne on sporangiophores arising directly from substrate mycelium; rhizoids and stolons absent	*Mucor*	
3b Rhizoids and stolons present		4
4a Sporangiophores usually borne opposite rhizoids and typically unbranched. Sporangia blackish	*Rhizopus*	
4b Sporangiophores often not opposite rhizoids, much branched. Sporangia not blackish	*Actinomucor*	

Key 2 Identifying species of *Rhizopus* involved in food fermentations

Identifying characteristics	Genus	Or see characteristic number
1a Rhizoids and sporangiophores delicate; sporangiophores up to 1 mm in height		2
1b Rhizoids and sporangiophores well developed; sporangiophores 2–4 mm high; good growth at 37°C; spores 7–9 μm in length	*R. oryzae*	
2a Spores spherical to globose in young cultures	*R. chinensis*	
2b Spores never globose or spherical in young culture		3
3a Spores 7–12 μm in length	*R. oligosporus*	
3b Spores 5–7 μm in length	*R. arrhizus*	

white to pale yellow to grayish. Most species—all those involved in fermented foods—are heterothallic with zygospores brown to blackish borne between equal or nearly equal suspensors. The zygospores are not seen unless cultures are mated under proper conditions.

Fermented foods involve relatively few *Mucor* species in the *Sphaerosporus* and *Racemosus* Sections (50), with the great majority found in the latter. The

first section contains species with globose spores; the only species known in fermented foods *(M. dispersus)* was reported from sufu. *M. dispersus* Hagem has spores (50) that are 9–18 μm in diameter. The other species belong in the *Racemosus* section and have been described as *M. javanicus* Wehmer, *M. praini* Chodat et Nechitch, *M. cambodja* Chrzaczcz, *M. dubius* Wehmer, *M. cirinielloides* van Tieghem, *M. rouxii* (Calm.) Wehmer, and *M. racemosus* Fresenius. The section contains *Mucor* species with chlamydospores in the sporangiophores, with short oval sporangiospores. The colonies age to gray or brown. Sporangiophores are strongly divided, bearing sporangia with walls breaking or only slowing deliquescing. Zycha & Siepmann (1969) recognize *M. racemosus, M. praini, M. javanicus, M. rouxii,* and *M. circinelloides. Mucor dubius* is considered a synonym of *M. javanicus,* and *M. cambodja* is cited as a possible synonym of *Rhizopus arrhizus.*

On the other hand, Schipper (42) recognizes only *M. circinelloides* and *M. racemosus,* placing *M. javanicus, M. dubius,* and *M. praini* as synonyms of *M. circinelloides.* The type culture of *M. praini* was seen, but *M. rouxii* and *M. cabodja* were not treated. Wehmer questioned whether *M. dubius* was a distinct species even as he described it. Therefore, at most, we have five species of *Mucor* in the Section *Racemosus* to consider, and these may be recognized according to Key 3.

AMYLOMYCES CALMETTE   This mucoraceous fungus exists, as far as is known, only in Chinese yeast, ragi, and similar products in the Orient. It is

Key 3 Identifying genera of *Mucor* involved in food fermentation

Identifying characteristics	Genus	Or see characteristic number
1a Sporangiophores repeatedly branched with many small sporangia borne circinately, especially near the surface of the agar; sporangiospores 3.7–9.5 × 3–7 μm	*M. circinelloides*	
1b Without sporangia borne circinately		2
2a No growth at 37°C; chlamydospores always very numerous in the sporangiophores	*M. racemosus*	
2b Growth at 37°C or if no or poor growth at 37°C then chlamydospores only occasionally seen		3
3a Colonies low, 1.0 mm sometimes to 0.6 cm in height	*M. rouxii*	
3b Colonies exceeding 1 cm in height		4
4a Sporangia to 70–90 μm; growth poor at 37°C	*M. praini*	
4b Sporangia 16–60 μm in diameter; growth good at 37°C	*M. javanicus*	

conceivably a mutant of *Rhizopus* that has been selected over the centuries to occur only in these starter preparations. *Amylomyces* is monotypic (14) and the sexual stage is not known, but in our laboratory many isolates from various regions have been mated, with negative results, both among themselves and with *Rhizopus*. All strains produce sterile white to light gray-brown colonies. The latter possess many abortive sporangia resembling aborted *Rhizopus* sporangia because of the black sporangia and adjacent region of the sporangiophore. Sporangiospore differentiation varies from none to sporangiospores that are irregularly released from the columella. The one distinct characteristic is the enormous number of chlamydospores produced in the aerial and substrate mycelium. Mature colonies when touched feel rotten because the mycelium fractures at the juncture of chlamydospores. These chlamydospores are thick-walled, smooth, cylindrical, oval to globose, generally intercalary, and measure 12–55 × 8–30 μm. The strains grow at 40°C but not at 45°C. Sporangiospores when formed are irregular in shape and size and often are not freed from the sporangium. Strains utilize sucrose, maltose, and glycerol and form lactic acid from glucose. All strains actively utilize soluble starch.

ACTINOMUCOR SCHOTAKOWITSCH   As far is known, the monotypic genus *Actinomucor* is only known to be used in the sufu fermentation, in which tofu is surface molded with *A. elegans* (Eidam) Benjamin and Hesseltine. Later the tofu cubes are placed in brine and the mold is killed. The proteolytic enzymes are released in the brine and then act upon the soy protein. The genus often is confused with *Mucor*, because it forms white to light buff *Mucor*-like colonies. Unlike *Mucor*, it possesses rhizoids and stolons, and differs from *Rhizopus* by having light-colored sporangia, lacking an apophysis (swelling of the sporangiophore just below the sporangium), and has branching sporangiophores, often in whorls. Thus, *Actinomucor* occupies a position intermediate between *Mucor* and *Rhizopus*. The sporangia are covered with conspicuous spines and contain many globose sporangiospores measuring 6–8 μm in diameter. The sporangia on the branches of the sporangiophores have persistent walls. The genus is distributed worldwide and is found in soil and on plant material. It can readily use L-xylose, starch and, in most instances, sucrose. Inorganic nitrogen sources are utilized. For a detailed description and a discussion of the genus, see Benjamin & Hesseltine (4).

ABSIDIA VAN TIEGHEM   *Absidia* appears to be associated with certain Chinese starters for beverage alcohol production. The only species involved belong to the *A. ramosa-corymbifera* group, which have been described in detail (13). *A. corymbifera* (Cohn) Sacc. et Trotter has been isolated repeatedly from yeast starter for the kaoliang wine fermentation, bran and wheat koji in China, and koji in Korea. Examination of the inoculum cake used in making

kaoliang wine always shows the presence of this *Absidia* species. Colonies of *A. corymbifera* are gray and grow and sporulate up to 37–45°C, depending on the strain. Stolons typically end in large pyriform sporangia with whorls of short sporangiophores below. The sporangiospores are smooth-walled, short oval, and measure 3–6.5 × 2.5–5 μm. Whether this species is essential in the above fermentation is unknown.

Ascomycetes

ASPERGILLUS MICH. EX FR.   Species of *Aspergillus* are commonly used in the Orient to produce koji. The function of the koji is primarily as a source of enzymes to act upon the starch, fats, and protein in the fermenting substrate. *Aspergillus* probably is used because of its higher tolerance to temperature, whereas species of *Penicillium* are never used in any of the Asian processes we know about because they cannot compete at high temperatures. The standard reference text to the taxonomy of *Aspergillus* is the monograph of Raper & Fennell (40). This treatment splits the genus up into 18 groups. We are concerned with only 4 of these, but members of the other 14 may be seen as contaminating molds in some foods produced by fermentation. The 4 are represented by species belonging to the *A. glaucus, flavus, niger,* and *wentii* groups. The first typically grow on substrates low in moisture, such as the later stages of the fish fermentation product, katsuobushi. The *A. flavus* group, the most widely used group, contains both *A. oryzae* (Ahlburg) Cohn and *A. sojae* Sakaguchi and Yamada. The group consists of species with greenish or greenish-yellow conidia. Only known, tested strains should be used because two other species in the group, *A. flavus* and *A. parasiticus* Speare, are producers of aflatoxins, a very potent group of carcinogenic mycotoxins. The *A. niger* group is characterized by black or nearly black spores. *Aspergillus wentii* Wehmer possesses yellow or yellow-brown conidia. For keys to identifying useful species within these groups, refer to Raper & Fennell (40).

MONASCUS VAN TIEGHEM   The only use of this fungus is in the production of a red pigment for coloring foods and alcoholic beverages by certain strains of *Monascus purpureus* Went. The most recent taxonomic account of the genus is by Iizuka & Lin (29). All species produce exogenous conidial chains that do not arise from sterigmata or metulae. Perithecia are found in a loose mass of mycelium, typically in the aerial mycelium. The perithecia produce numerous ascospores that are released upon the rupture of the peritheicial wall. Iizuka & Lin recognize 12 species and 2 varieties. The classification is based on sexual reproduction on potato dextrose agar, the colony appearance, the shape of the conidial chains, the production of pigment, and the tolerance to 30% ethanol and to 6% NaCl. In their scheme, *M. purpureus* forms no peritheica on potato

dextrose agar, has spiral chains of conidia, produces pigment, and cannot grow in 30% ethanol and 6% NaCl. Some of the other species they recognize may also be used in production of pigment.

NEUROSPORA SHEAR ET DODGE   This genus is used in Indonesia only in the fermentation of peanut press cake or of soybean residue made into an orange-colored cake called ontjom. The genus *Neurospora* is quite unrelated to the other ascomycetes (e.g. *Monascus* and *Aspergillus* used in fermented foods. *Neurospora* is typically a very rapidly growing fungus with both homothallic and heterothallic species, with the genus placed in the Sordariaceae in the order Sphaeriales. *Neurospora* has persistent perithecia with black one-celled ascospores that are shot away from the perithecium through an ostiole at maturity. According to Ho (28), who examined 71 cultures, the fungus used in ontjom production is *Neurospora intermedia* Tai, not *N. sitophila* Shear and Dodge as originally reported. *Neurospora intermedia* is heterothallic, producing perithecia containing asci with 8 ascospores per ascus. The conidial stage is bright yellow to apricot or carrot red, whereas *Neurospora sitophila* has pinkish conidia. Also, the conidia of *N. intermedia* are larger than those of *N. sitophila*. In crosses with *N. crassa* Shear and Dodge, *N. sitophila* and *N. intermedia*, fertile perithecia were formed only with authentic *N. intermedia* isolates. The *intermedia* strains produced appreciable amounts of extracellular amyloglucosidase. A recent extensive study by Perkins et al (39) of *Neurospora* strains (675) collected all over the world showed *N. intermedia* to be abundant in Asia. All the strains from ontjom were members of this species. According to these authors, the best way to identify heterothallic species of *Neurospora* is to mate the unknown with mating types of known species.

YEASTS   "Yeast" is a broad, nonscientific name for fungi that in all or part of their vegetative lives exist as single cells. Many species are ascomycetes, some are probably ascomycetes in which the sexual state is unknown, and still others, such as *Rhodotorula* Harrison, are the yeast phase of Basidiomycetes. As far as can be determined, all the yeasts involved in Oriental fermented foods belong to the first two groups, and therefore are probably ascomycetes.

The genera of yeasts reported to be used in Asia to prepare fermented foods belong to *Saccharomyces* Meyen emend. Reess, *Saccharomycopsis* Schionning, *Torulopsis* Berlese, *Trichosporon* Behrend, *Candida* Berkhout, *Endomycopsis* Dekker, and *Pichia* Hansen (*Hansenula* H. et P. Sydow). For those yeasts occurring in fermented foods in India, the best and most recent study is that of Batra & Millner (3).

Endomycopsis burtonii Boidin, Pignal, Lehodey, Vey et Abadie   This species, one of the two yeasts isolated by Djien from ragi (10), is characterized by

producing true mycelium with blastospores, and ascospores that are spherical, hat-, saturn-, or sickle-shaped. Nitrate is not assimilated, but galactose, sucrose, and maltose are. This heterothallic yeast was first isolated from rice ferment (37), and other strains have come from ragi.

Hansenula anomala (Hansen) H. et P. Sydow   This yeast produces hat-shaped ascospores; ferments glucose, sucrose, and maltose weakly or not at all; assimilates inulin; and does not require an exogenous source of vitamins. In Lodder's book (37), 10 strains of the 117 studied were isolated from ragi. Batra & Millner (3) report isolation of this species from murcha (like ragi but produced in Nepal and India) and in kanji (a beerlike beverage made in India from carrots and beets with spices). In Delhi, this yeast was the only species present in kanji.

Zygosaccharomyces rouxii (Boutroux) Yarrow (Saccharomyces rouxii Boutroux)   This heterothallic yeast is common in high-salt substrates, and is therefore used in the miso and shoyu fermentations. It is also found in jams and syrups. Of all the yeasts described from fermented foods, this one is best established as an essential microorganism. According to Lodder, it has been described under many names, such as *S. soya* Saito and *Zygosaccharomyces japonicus* Saito. This osmophilic yeast grows on 60% w/w glucose-yeast extract agar; raffinose, sucrose, galactose, and melibiose are not fermented, but maltose is. Ascospores usually are produced only when freshly isolated strains of opposite mating types are combined; the resulting ascospores are one to four per ascus and are spheroidal to ellipsoidal.

Saccharomyces cerevisiae Hansen   This common species has been reported from various fermented foods. Batra & Millner reported its occurrence in papadam (a condiment) and janjabi waries (fermented leguminous grain paste), both in India. Like other species of *Saccharomyces*, vegetative reproduction is solely by multilateral budding with no true mycelium. Ascospores are spherical and number one to four per ascus. Cellobiose and salicin are not utilized; soluble starch is not fermented, but raffinose, sucrose, maltose, and galactose are.

Candida etchellsii (Lodder et Kreger-van Rij) Meyer and Yarrow (Torulopsis etchellsii Lodder et Kreger-van Rij)   This species is osmophilic, being isolated from fermenting cucumber brine, lemon concentrate, and shoyu fermentation. According to Yokotsuka (48), *Torulopsis etchellsii* and *T. versatilis* contribute to the flavor in shoyu. It ferments only glucose and maltose and does not form pseudomycelium. It assimilates nitrate, glucose, and maltose but not

sucrose, lactose, cellobiose, or trehalose. Characteristically for the genus, it does not produce ascospores and has multipolar budding.

Candida versatilis (Etchells et Bell) Meyer et Yarrow (Torulopsis versatilis (Etchells et Bell) Lodder et Kreger-van Rij)   This species is very similar to *C. etchellsii* except that it assimilates lactose and ferments trehalose. This yeast also grows in shoyu and cucumber brine.

Recently Yarrow & Meyer (47) have transferred the two *Torulopsis* species above to the genus *Candida*, as *C. versatilis* and *C. etchellsii*. They found it impractical to separate *Candida* and *Torulopsis* on the basis of presence or absence of pseudohyphae.

Candida famata (Harrison) Meyer et Yarrow (Torulopsis candida (Saito) Lodder)   A third species has been reported from idli (a fermented dough product of North India made from rice and black gram flours). It assimilates cellobiose, glucose, D-xylose, sucrose, and maltose but does not assimilate nitrate and does not ferment lactose. It is apparently not found in high-salt brines.

Trichosporon pullulans (Lindner) Diddens et Lodder   This species always has true mycelium and arthrospores but has no ascospores. This yeast has been reported from idli by Batra & Millner (3). It assimilates lactose and nitrate but does not cause fermentation on any substrate. According to Lodder (37), it is often isolated in connection with breweries.

Saccharomycopsis malanga (Dwidjoseputro) Kurtzman, Vesonder, and Smiley   This is an important food yeast of the Orient, having been isolated repeatedly from ragi and Chinese yeast. It is represented by a number of isolates in the ARS Culture Collection at the Northern Regional Research Center. Originally described as *Hansenula malanga* in isolates from ragi (36), it is characterized by the extracellular production of 3-d-hydroxy-palmitic acid, which is not encountered in *S. fibuligera*. It contains two hat-shaped ascospores and produces asci that often rupture at maturity. Fermentation of glucose is slow and weak, as it is also on maltose. It is separated from a second species of *Saccharomycopsis*, *S. fibuligera*, because it does not assimilate sucrose.

Saccharomycopsis fibuligera (Lindner) Klocker   The original isolate of this species was made from "chalky bread." Strains have been isolated from Chinese yeast, ragi, and tape. It assimilates sucrose and also maltose, cellobiose, and soluble starch. Ascospores are hat-shaped with two to four spores per ascus. Often no conjugation of cells is observed. Characteristic of the genus

is the production of long oval to cylindrical vegetative cells with pseudomycelium.

Although the yeasts appear to be the important ones in Oriental food fermentation, there may be others misidentified or not yet reported.

Bacteria

LACTIC BACTERIA   Because of the lack of any detailed study, the role of lactic bacteria in oriental fermentations is poorly known. Traditionally, investigations have focused on the mold and yeast components rather than the bacterial flora. Recent studies on ragi, however, found bacterial counts of several million per gram of what appears to be the same bacterium—even though the ragi starter is extremely dry, like wheat flour. In the Japanese miso and shoyu fermentations, the bacterium is reported to be *Pediococcus halophilus* Mees. This species grows best in 6–8% NaCl and tolerates 15%. My feeling, based on fragmentary evidence in the literature, is that the bacteria (except for *Bacillus subtilis* as below) are restricted to the genera *Streptococcus, Leuconostoc, Pediococcus*, and *Lactobacillus*, and to only a few species in each of these genera. For a simple method of identifying these lactic acid bacteria,see Key 4.

The idli fermentation in India is caused at least in part by *Leuconostoc mesenteroides* (Tsenk.) van Tieghem. According to Mukherjee et al (38), this is one of several bacterial species present in idli. The authors also found *Streptococcus faecalis* Andrews and Horder and *Pediococcus cerevisiae*

Key 4   Identifying lactic acid bacteria involved in food fermentations[a]

Identifying characteristics	Genus	Or see characteristic number
1a Cells as rods, usually occurring as single cells or in chains; anaerobic or facultative; produce lactic acid	*Lactobacillus* Beijerinck	
1b Cells spherical or ovoid in pairs or chains		2
2a Homofermentative, yielding chiefly dextrorotary lactic acid; cell division in one plane—hence, cells occurring in pairs or chains; catalase negative	*Streptococcus* Rosenbach	
2b Homofermentative, yielding inactive lactic acid; cell divisions in two planes resulting in pairs and tetrads of cells; catalase variable	*Pediococcus* Balcke	
2c Heterofermentative, yielding levorotary lactic acid, CO_2, ethanol, and/or acetic acid; cell division in one plane resulting in pairs or chains of cells	*Leuconostoc* van Tiegh	

[a]Modified from (6)

Balcke in this fermentation. *Leuconostoc mesenteroides* and *S. faecalis* appeared early in the fermentation, followed by high acid-producing *P. cerevisiae*. This is typical of many bacterial fermentations in which no pure inoculum is used and in which the substrate is sufficiently wet for growth of bacteria. In all the Asian fermentations, the result is an acid type of food. Probably many dairy-type lactics are present in fermentations in which milk of cattle or water buffalo is incorporated with plant material.

BACILLUS COHN   Only one species of *Bacillus* is involved in food fermentation, *Bacillus natto* Sawamura. This organism is used for making natto, a solid product made with whole soybeans. However, this species is considered to be a synonym of *B. subtilis* (Ehrenberg) Cohn according to Gordon et al (17), who examined several strains identified as *B. natto. Bacillus subtilis* is commonly found on soybeans. It is characterized by oval cells, is grown aerobically, has motile cells, and grows up to 45°–55°C. Heat-resistant spores are formed that are ellipsoidal or cylindrical, central or paracentral in the cell, and usually not distending the sporangial wall. It is catalase positive, growing in 7% NaCl and down to pH 5.7. Probably not all strains of *B. subtilis* are suitable for making good natto.

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